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## **TISSUE CULTURE STRATEGIES FOR GENETIC IMPROVEMENT OF BAMBOO**

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### Abstract

Bamboos are among the economically most important plants world-wide. In Europe bamboos are used as ornamentals for gardens, but there is increasing interest for uses in ecological applications and as energy crops. Biotechnological techniques, including tissue culture, in vitro hybridisation, molecular markers and genetic transformation are crucial for the future of bamboo.

Micropropagation of bamboos has allowed to develop a new type of ornamental bamboo that can be produced yearround with a high quality / price ratio and distributed far more widely than classically propagated ornamental bamboos. Molecular markers are used in quality control procedures.

Flowering of bamboos is still one of the greatest mysteries in botany, and breeding systems are non-existent. However, flowering can be induced reproducibly in tissue culture, both in seedlings and in adult bamboos, providing the only method for hybridisation. The flowering structures that are used are pseudospikelets, morphological features unique to the subfamily of Bambusoideae. These special propagules can be used for propagation, long term storage, for hybridisation and for genetic transformation. While flowering can be induced, controlled and reversed in tissue culture, a more fundamental approach to unravel the mechanisms of flowering include studies of cell division patterns and profiles of volatile components.

Some applications of biotechnology for bamboo are presented, with emphasis on research strategies in a SME. Furthermore, all the techniques developed are of use not only in horticulture, but also in agriculture and forestry worldwide.

### 1. Introduction

Bamboo is an ornamental plant, mainly for garden use in Europe. Besides, two different pathways for valorisation of biotechnological research have emerged: (1) in the tropics bamboo is a very important plant, providing livelihood for over 500 million people and providing housing and shelter for over 1 billion people; (2) “Bamboo for Europe”, an EU-funded research project has allowed to develop the potential of bamboo as an agricultural plant in Europe (Gielis and Oprins, 2000) with possible applications in wood industry (Van Acker *et al.*, 2000) and as energy crop. So, any biotechnological developments for the improvement of ornamentals will also be useful on a much larger scale.

To develop a commercially feasible process of bamboo propagation and genetic improvement three major components are important: (1) fundamental research on bamboo physiology and genetics, including the development of tissue culture techniques for hybridisation of bamboo; (2) the development of axillary branching into a universal technique with a high clonal fidelity and very high efficiency; (3) forward integration to optimise the added value of micropropagation. This article focuses specifically on the development of micropropagation systems, on the use of molecular markers as part of quality control and on research on flowering in tissue culture of bamboo.

## 2. Micropropagation of bamboo

### 2.1. Research on bamboo micropropagation

For bamboo different propagation techniques are available, such as seed propagation, clump division, rhizome and culm cuttings (Banik, 1994; Banik, 1995). But these methods suffer from serious drawbacks for large or mass scale propagation. For mass scale propagation (> 500 000 plants per year) classical techniques are largely insufficient and inefficient, and tissue culture is the only viable method. Indeed, the order of magnitude of the demand for bamboo planting materials indicates that micropropagation will inevitably be necessary for mass scale propagation (Subramanlam, 1994; Gielis, 1999).

By now a large number of papers on micropropagation (the use of tissue culture for propagation only) of bamboos have been published, original papers as well as reviews and some in which tissue culture is described in a more general aspect. Many researches have focussed on somatic embryogenesis of seedlings of tropical bamboos (INBAR, 1991). An inventory (BIC, 1994) shows that at least 21 labs in South-east Asia were involved in bamboo tissue culture, mainly in India (reviews on tissue culture of bamboo can be found in INBAR, 1991; Saxena and Dhawan, 1994; Zamora, 1994; Nadgauda *et al.*, 1997a; Gielis, 1999). Major research focused also on the clonal propagation of elite genotypes, either juvenile or adult. The number of papers about this subject however is much less, and this is solely due to lack of success. Indeed, technically the propagation of adult plants via axillary branching is much more difficult than with seedlings of tropical bamboos.

For tissue culture of bamboo the use of starting material (seeds or adult plants) and the choice of the propagation method are crucial (Gielis, 1999). The two major advantages of using seedlings are that seedlings establish a new generation, and that the technology is easier. But the disadvantages are considerable: (1) insufficient or no knowledge of genetic background, (2) restricted availability of seeds for most species and rapid loss of germination capacity, and (3) comparison of *in vitro* to *in vivo* performance has not been thoroughly evaluated. In addition there is a huge variability in responsiveness in tissue culture (Saxena and Dhawan, 1994).

When using adult bamboos main problems are: (1) endogenous contamination, (2) hyperhydricity and instability of multiplication rates, and (3) many problems with rooting also in bamboos that root readily in nature. Rooting percentages for adult bamboos ranged from very low percentages of 10% for *Bambusa vulgaris* to 73% for adult *Dendrocalamus longispathus* (Saxena and Dhawan, 1994). A rooting percentage of 77% was obtained for adult *Dendrocalamus giganteus* in 3 or 4 weeks (Ramanayake and Yakandawala, 1997). But, while 77% of success is good on 500 plants in a laboratory experiment it still represents a loss of 33000 when you transplant 100 000 plants. Low

rooting frequencies are the major bottleneck to developing commercially viable protocols (Saxena, 1993). The combination of photomixotrophic *in vitro* multiplication and photoautotrophic *in vitro* rooting stages resulted in improved transplanting success (Watanabe *et al.*, 2000). Improvement of rooting percentages and transplanting has been achieved in various commercial laboratories.

## 2.2. Commercial micropropagation of bamboo

Micropropagation via tissue culture thus attracted a lot of attention but the translation and transformation of these expectations into commercially viable propagation systems has been beset with a number of problems that were either technological, or related to marketing. At present only a very limited number of laboratories specialise in the production of bamboos at a commercial scale. Oprins Plant, Belgium, currently has over 60 genotypes in culture initiated from adult genotypes of which about 30 on a commercial scale (Gielis and Oprins, 1998). Species and varieties of *Arundinaria*, *Chimonobambusa*, *Fargesia*, *Phyllostachys*, *Pleioblastus*, *Sasa*, *Sasaella*, *Semiarundinaria*, *Shibataea* and *Yushania* (temperate bamboos) and *Bambusa*, *Dendrocalamus*, *Dinochloa*, and *Thyrsostachys* (tropicals) are produced commercially. Rooting percentages of most species are between 85 and 100%, depending on species and season of transplanting (unpublished results). These bamboos are produced either as ornamental, or for tropical forestry. Other laboratories focusing on bamboo tissue culture include West Wind Technology, USA, and Bamboo World, Australia, (Cusack, 2000). Several other laboratories such as Piccoplant, Germany, and Microflor, Belgium, produce a more limited number of bamboo species as part of their tissue culture operation.

Other laboratories initially involved in tissue culture of bamboos however, no longer produce bamboo commercially, for example TERI, India (S. Saxena, pers.comm.) and Thai Orchids Lab, Thailand (P. Gavinlertvatana, pers.comm.). The main problem is that micropropagation of bamboos also has to deal with the development of new markets and market opportunities. Logistics and supply chains such as in ornamental horticulture where tissue culture labs are separate entities seem not to work. Instead the laboratories have to develop their own markets, marketing and sales for bamboo plants (Gielis and Oprins, 1998). This strategy of forward integration needs to go beyond the classical scheme of tissue culture. The tissue culture phase terminates at Stage III, but in regard to marketing of plants one can also distinguish subsequent stages: (1) Stage IV, the transplantation stage with the end product being rooted plantlets in trays, (2) Stage V, the production of liners, either for production of saleable plants or for use as micromotherplant, and (3) Stage VI, the production of saleable plants. This distinction is important if the complete chain of production is integrated in a single company, since this determines the added values. In our case the laboratory produces ornamental plants exclusively for Oprins Plant's own nurseries. A balanced production and logistic chain, where bamboos are produced in Belgium and grown in the nurseries in Spain and France, has allowed to produce high quality ornamental bamboos year round at prices which are 40-60% below the current market prices for bamboo. These plants are currently being sold under the brand name BambooSelect™, which is an additional tool in marketing. In an agricultural perspective it is now possible to produce the same genotypes for large scale plantations in Europe at prices comparable to other energy crops (Gielis and Oprins, 2000).

### 3. Monitoring genetic stability in bamboo tissue culture

#### 3.1. AFLP markers in quality control of micropropagation

Somatic mutations in bamboo are highly valued in horticulture. In various bamboos somatic mutations are known that alter the stem colour or stem shape, such as *Bambusa ventricosa* with inflated internodes (Ohrnberger, 1999). But not all are stable in tissue culture. The bulbous internodes of *Bambusa ventricosa* were lost after tissue culture (Huang and Huang, 1995) and variegation of stems and leaves disappears very often in tissue culture. Tissue culture has been associated with molecular aberrations in general (including also chromosomal defects), and phenotypic defects may show up only some years later. Therefore it is important to devise methods of quality control at the tissue culture stages to minimise the potential danger of such defects, for example when plants of Stage VI are sold in large quantities.

Generally it is very difficult to find precise defects, especially when somatic mutations are possibly linked to transposon activity. In a first approach AFLP™ markers are used to assess the stability and clonal fidelity of tissue cultured bamboos. In one experiment *Phyllostachys* species and cultivars were collected 1) from the garden of Dr. Jacques Van Dooren, Kuntich, reference collection of bamboo for Belgium (see also Gielis *et al.*, 1997a), 2) from the tissue culture lab at Oprins Plant and 3) from plants transplanted in the greenhouse. Using this set up, it should be possible to detect if genotypes in the collection and in propagation have the correct names, and whether the plants cultured are subject to considerable clonal variation.

*Phyllostachys*-genotypes sampled in the laboratory included *P. aurea*, *P. aureosulcata* 'Spectabilis', *P. aureosulcata* 'Aureocaulis', *P. bissetii*, *P. humilis*, *P. nigra*, *P. nigra* 'Henonis' and *P. vivax*. Of all these types, except *P. nigra*, samples were also taken in the greenhouse of hardened tissue cultured plants. In Table 1 code numbers, names and provenances are listed that were used in the experiments. AFLP analysis was performed according to Vos *et al.*, 1996, although special care was taken to sample young leaves from the collection (Gielis *et al.*, 1997b). One primer-enzyme combination was used with different selective nucleotides. The whole set of bamboos was screened on 71 polymorphic markers (Quantas software, Keygene, Wageningen)

The dendrograms of Figure 1 show that most bamboos in tissue culture and transplanted in the greenhouse group together with the collection plants. The exception is *Phyllostachys vivax* from the laboratory that turns out to belong to the *nigra* group, while the collection plant of *P. vivax* does not group at all with the *nigra*'s. This is a clear case of mistaken identity, due to mislabelling of the motherplants obtained from China. If one observes the dendrograms it is seen that all *P. aurea* cultivars, all *P. nigra* cultivars, and all *P. aureosulcata* cultivars group together. They are, according to this test, 100% identical. However, they differ in stem colour, leaf colour, habit etc, and these differences can be observed readily in the field. This also means that the method used is not sensitive enough to detect those differences.

But it clearly shows that AFLP is a useful method when used as part of quality control in the tissue culture process, to assess correct clonal identity. This can then be used towards customers. AFLP markers are now used routinely besides other measures to ensure clonal fidelity. These other measures include the use of axillary branching as propagation method and annual initiation of new cultures, so that the maximum number of subcultures is 12 to 14.

### 3.2. The search for the molecular basis of somatic mutations in bamboo

Based on phenotype and mode of action somatic mutations involved in stem colour of bamboo are possibly caused by transposon activity. The study of transposon activity and their possible relation to certain mutations is very difficult. In bamboo no breeding systems are available to detect transposon activity through classical breeding. Molecular approaches may provide some shortcuts, but to link the presence and activity of transposons to certain phenotypic markers is again a difficult task. A first approach to identify the molecular basis of these somatic mutations was to use primers based on sequence information from the 4.5 kb Ac9 transposon from maize. Several primer combinations were used and several copies with considerable homology to the original Ac9 transposons were present (Gielis and Sormann, 1997), which eventually turned out to be a useful and relatively cheap method to identify bamboos, at the species level.

Primers designed to detect *Activator*-like transposons in *Petunia* could also be used to detect fragments in bamboo (De Keuckeleire, 2000). The same fragment was detected in the three bamboos tested, namely *Bambusa vulgaris*, *Sasa veitchii* and *Phyllostachys edulis*. The fragment of *Bambusa vulgaris* (*hATbv1*) was sequenced (De Keuckeleire, 2000) and shown to be homologous to members of the *hAT* superfamily of transposons, to which also *Ac* (corn), *Tam3* (*Antirrhinum majus*) and *hobo* (*Drosophila*) belong. The homology was as high as 60% in some regions, which conforms also to earlier findings of Ac-like sequences in *Bambusa multiplex* (Huttley *et al.*, 1995). It is very likely that many other transposons are present in the bamboo genome as well.

A next step is to use these AFLP-primers in combination with methylation sensitive AFLP, in which methylation sensitive restriction enzymes are used to cleave sites within the bamboo transposon. The relation between methylation patterns and transposon activities is well established (Martienssen, 1998) and the activation of transposons during tissue culture is also a well documented phenomenon. The use of genotypes that have been cultured for six, eighteen, twenty-four and thirty-six months should allow to assess if long term tissue culture has any influence on methylation in general and on methylation patterns of transposons. This research is currently ongoing.

### 4. Flowering of bamboo in tissue culture

Because of the peculiar flowering habits in bamboo it has been almost impossible to breed for superior traits in woody bamboos. The first reports on tissue culture flowering of bamboos (Nadgauda *et al.*, 1990; Rao *et al.*, 1990) caused great excitement. It opened up the possibility of controlled flowering that can be used for breeding of bamboo. Since then *in vitro* flowering has been observed in many types of bamboos, both in seedlings and mature bamboos (Gielis and Debergh, 1998; Gielis, 1999).

More than 10 years after the first report on *in vitro* flowering in bamboo some positive results have been obtained, but practical and commercially exploitable results have not been reported yet. The most promising potential is for hybridisation under controlled conditions (Rao and Zamora, 1995). But many hurdles still need to be taken before the methods really become applicable at agricultural scales. Recently a new research project was initiated in our group to improve the induction of flowering and the quality of flowering parts, in order to be able to hybridise bamboos in the future. While

this project aims mainly at developing techniques, it also includes various approaches on a more fundamental level. The use of pseudospikelets is central in this research.

Pseudospikelets occur only in bamboos, not in other grasses. In grasses a spikelet bears two empty glumes, but the glumes of pseudospikelets in bamboos subtend dormant buds that can develop into new pseudospikelets. The proximal parts of the pseudospikelets do not develop into flowers but in tissue culture conditions keep on multiplying indefinitely (Figure 2; Gielis, 1999), allowing to establish monocultures of pseudospikelets. Flowers are formed in the distal parts of individual pseudospikelets (unpublished results) and do not develop to anthesis under normal tissue culture conditions. They can however, be forced to develop fully under more adverse conditions. Cultural conditions can control flowering *in vitro* to a large extent (Nadgauda *et al.*, 1997a, 1997b; Gielis, 1999). Factors used for the induction of pseudospikelets are mainly cytokinins (Nadgauda *et al.*, 1997a; Joshi, pers.comm.). However, cytokinins are involved in various flowering processes and more fundamental research is urgently needed. Unfortunately, despite the huge potential of tissue culture flowering of bamboo, to our knowledge only one other laboratory is actively engaged in this field of research (Dr. Nadgauda at National Chemical Laboratory in Pune, India).

Despite the presence of flowers in the axils of pseudospikelets, dormant buds can develop into vegetative shoots. Pseudospikelets can also be regarded as highly contracted axes, in which leaves are reduced to glumes (Figure 2). Instead of propagules and plantlets of 2 to 5 cm in tissue culture, pseudospikelets have lengths between 0.25 and 1.5 cm. This is a miniaturisation of the plants and allows a good opportunity to use these pseudospikelets for propagation of bamboos. With these smaller clumps, operators can cut and transfer much easier, while the mode of propagation is still via axillary branching, thus the method with lesser risk for somaclonal variation. Since leaf area is much reduced, quality problems in the propagation stage are much less important. This allows for a considerable cost reduction in micropropagation, since operator costs represent 75-80% of cost price of tissue culture plants.

In tissue culture systems for propagation flowering of bamboo represents considerable risks since many bamboos flower monocarpically in nature and die completely after flowering. It is very important to be able to control flowering and reversion of flowering of bamboo. Therefore the influence of tissue culture flowering on vegetative growth *post vitro* has been studied extensively in three independent experiments (unpublished results). (1) plants of *Bambusa tuldooides* regenerated from pseudospikelets grow as vegetative plants after hardening; (2) In large scale vegetative propagation only two flowering plants of *Phyllostachys* have been observed after transplanting about 500000 plants, and (3) tests with *Sasa palmata* have shown that even when flowering plants are transplanted in the greenhouse, these revert to the vegetative state within a few weeks. These tests have shown that tissue culture technology does not lead to monocarpic flowering of saleable plants.

Using pseudospikelets as propagules we have now a system which can be used for propagation and for the induction of flowering and flowering structures. The same propagules can also be used for long term storage and for genetic transformation.

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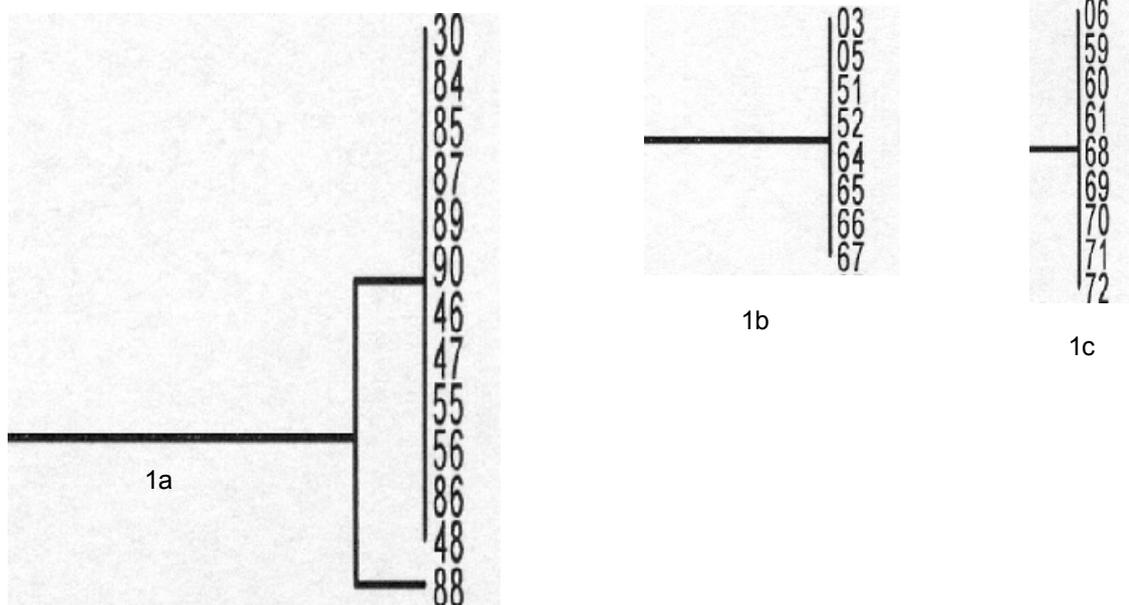


Figure 1: Parts of the dendrogram of *Phyllostachys* tests based on 71 polymorphic markers. Fig. 1a is the *P. nigra* group, 1b *P. aurea*-group and 1c *P. aureosulcata* group. As can be seen in all figures the methodology used cannot discriminate between cultivars and forma.



Figure 2: A clump of pseudospikelets of *Bambusa ventricosa*. On the left a vegetative shoot has developed.

Table 1: Bamboo species and cultivars and their provenances used in AFLP experiments.

code	Species and cultivar	collection	In vitro	Greenhouse Stage V
30	<i>nigra</i> 'Henonis'	*		
84	<i>nigra</i>		*	
85	<i>nigra</i> 'Henonis'	*		
87	<i>nigra</i> 'Punctata'	*		
89	<i>nigra</i> f. <i>megurochiku</i> '	*		
90	<i>nigra</i> 'Fulva'	*		
46	<i>nigra</i>			*
47	<i>nigra</i> 'Henonis'		*	
55	<i>vivax</i>		*	
56	<i>vivax</i>			*
86	<i>nigra</i> f. <i>nigra</i>	*		
48	<i>nigra</i> 'Henonis'			*
88	<i>nigra</i> 'Boryana'	*		
03	<i>aurea</i>	*		
05	<i>aurea</i>	*		
51	<i>aurea</i>		*	
52	<i>aurea</i>			*
64	<i>aurea</i>	*		
65	<i>aurea</i> 'Holo-chrysa'	*		
66	<i>aurea</i> 'Koi'	*		
67	<i>aurea</i> 'Flavescens-inversa'	*		
06	<i>aureosulcata</i> 'Alata'	*		
59	<i>aureosulcata</i> 'Spectabilis'		*	
60	<i>aureosulcata</i> 'Spectabilis'			*
68	<i>aureosulcata</i> 'Spectabilis'		*	
69	<i>aureosulcata</i>	*		
70	<i>aureosulcata</i> 'Aureocaulis'	*		
71	<i>aureosulcata</i> 'Spectabilis'	*		
72	<i>aureosulcata</i> 'Harbin'	*		

